(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number: **0 073 554**
A1

(12) EUROPEAN PATENT APPLICATION

(21) Application number: **82302524.2**

(22) Date of filing: **18.05.82**

(51) Int. Cl.³: **G 01 N 33/50**, G 01 N 33/48
// G01N15/02, G01N15/06

(30) Priority: **26.06.81 US 277539**

(43) Date of publication of application: **09.03.83**
Bulletin 83/10

(84) Designated Contracting States: **BE CH DE FR GB IT LI NL SE**

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(54) Treatment of whole blood for determination of red blood cell volumes.

(57) Whole blood is treated to sphere, and optionally also fix, red blood cells in order to be able better to determine their volume and (optionally) number, by treating the blood with (a) an isotonic solution of a sphering agent, and (b) an isotonic solution of a sphering agent and a protein, to provide a weight ratio of protein to sphering agent of from 20:1 to 70:1, and a final concentration of sphering agent of from 2 to 10 mg/100 ml. In a modification of this procedure, use is made of the protein endogenous to the blood and step (b) is replaced by (c) treating the mixture from step (a) with an isotomic solution of a fixing agent such as glutaraldehyde. Reagent compositions comprising a sphering agent and a protein, for use in the above method, are also described.

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TITLE MODIFIED
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TREATMENT OF WHOLE BLOOD FOR DETERMINATION OF RED BLOOD CELLS

This invention relates to a method of treating mammalian whole blood to provide a sample suitable for electrooptical determination of red blood cell volumes, and more particularly to a method for sphering or sphering and fixing whole blood erythrocytes without volume change for accurate and precise cell volume measurement.

Known methodswhich utilize the measured amount of light scattered from individual red cells (erythrocytes) to determine the individual and mean volumes of red cells, suffer from two kinds of error:

1. The native human red blood cell is a biconcave disc and the amount of light scattered within a particular solid angle varies with the orientation of the cell with respect to the incident light beam.

2. During handling, e.g. dilution and pumping, the shape of the cells can change depending, in part, on the time between the drawing of the blood and the time of measurement and, in part, on the composition of the diluted blood sample.

For a discussion of the above, see Hemolysis and Related Phenomena, Chapter II, pp 10-49 by Eric Ponder (1948) and Transformation and Restoration of Biconcave Shape of Human Erythrocytes Induced by Amphiphilic Agents and Changes of Ionic Environment, Bichemica Et. Biophy. Acta, Bernard Deuticke, pp 494-500 (1968).

It is known, see for example Ponder supra, that it is possible to sphere red blood cells in isotonic solution without changing their volumes. Since the light scattering from a perfectly spherical cell is not dependent on its orientation in a light beam, the first kind of error can thereby be reduced or eliminated. However, such preparations are notoriously unstable and red cell lysis occurs at various times after sphering, depending on the choice of sphering agent and the properties of the individual blood samples.

We have now found that prolonged stability of the sphered state can be achieved by controlling the absolute concentration of the sphering agent (typically a material with detergent properties) and the weight ratio of sphering agent to protein in the mixture. The protein helps to ensure shape consistency during processing and minimizes the second kind of error.

According to a first aspect of the invention, there is provided a method of treating mammalian whole blood to provide a sample suitable for electrooptical determination of red blood cell volume, which comprises the steps of (a) combining an anticoagulated whole blood sample with a first isotonic solution containing sphering agent, and (b) treating an aliquot of the resulting mixture with a second isotonic solution containing protein and sphering agent; wherein the weight ratio of protein to sphering agent in the aliquot and in the final sample is from about 20:1 to about 70:1, and the concentration of sphering agent in the final sample is from about 2 mg./100 ml. to about 10 mg./100 ml.

The protein can be added to the blood sample in step (b) above or, alternatively, use can be made solely of the protein endogenous to the blood. In the latter case, the invention provides a modification of the above

method wherein instead of step (b), said aliquot from step (a) is treated in a step (c) with an isotonic solution containing fixing agent, and wherein the aliquot from step (a) has a weight ratio of endogenous protein to sphering agent of from about 20:1 to about 70:1, and a sphering agent concentration of from about 2 mg./100 ml. to about 10 mg./100 ml.

Thus, the method of this invention can be carried out generally in two ways:

A. A blood serum sample is diluted, typically about 1/1000, in an isotonic solution containing sphering agent (detergent) and a protein, preferably, albumin at the required concentrations; or

B. The blood serum sample is diluted with an amount of isotonic solution containing the sphering agent at a concentration which is just sufficient to cause sphering when the dilution provides the correct ratio of sphering agent to the endogenous serum albumin (plasma protein) from the blood sample itself. The resulting sample is then simultaneously and/or successively fixed and further diluted by adding an isotonic solution of a fixing agent to harden the sphered cells and make them completely insensitive to processes which could otherwise cause them to change their shape or size or lyse and lose their contained hemoglobin.

Preferably, prior to step (a) of the method of the invention, the whole blood sample is diluted with saline, as diluent, resulting in about a 50% by volume dilution. This reduces the viscosity and thus reduces the likelihood of volumetric pumping errors which stem from variations in blood sample viscosities. The subsequent dilution steps will then result in a final dilution of blood sample of about 1:1000 by volume, i.e. a dilution such that the probability of more than one cell passing through the incident light beam of an electrooptical detector during the detector's measuring time window is

very low.

The sphering agent (detergent) used in the method of the invention is preferably an alkali metal salt of an alkyl sulfate, the alkyl group containing from 10 to 16 carbon atoms. Sodium lauryl sulfate is most preferred. The preferred protein from addition to the blood sample is serum albumin.

In the modified method of the invention, the preferred fixing agent solution used in step (c) is an isotonic glutaraldehyde-containing saline solution.

In another aspect, the invention provides a reagent for sphering the red blood cells in an anticoagulated whole blood sample, which comprises a mixture of a protein and a sphering agent wherein the weight ratio of protein to sphering agent, when said reagent is mixed with a blood sample, is from about 20:1 to about 70:1, and wherein the total concentration of sphering agent is such as to provide, when said reagent is mixed with the blood sample, from about 2 mg./100 ml. to about 10 mg./100 ml. of sphering agent in the blood sample mixture.

When a sphering agent is added to a whole blood sample in the absence of added protein, the amount of free sphering agent in solution is dependent upon the concentration of red cells (see Ponder above). Thus, when a reagent containing a fixed optimal sphering agent concentration for a normal blood count is used (without protein), the degree of sphering could be either incomplete, with a blood with high red cell count per unit volume of solution, or could lead to lysis with a very low red blood cell count. Proteins, such as serum albumins, bind sphering agent reversibly and are thus used in accordance with the present invention to buffer the effective concentration of sphering agent in the optimal range, independent of red cell count.

The preferred concentration of sphering agent used in the present invention is that amount which is just

sufficient to cause sphering when buffered with a protein such as albumin or plasma protein at any particular dilution of sample. As stated previously, the protein albumin can be provided in either of two ways: by addition thereof to the blood sample, or endogenously as plasma protein in the serum sample.

In one preferred embodiment of this invention, the method involves combining a prediluted blood sample with an isotonic sphering agent-saline solution, and then treating an aliquot thereof with a protein-sphering agent saline solution, to achieve the concentrations defined above.

Preferably, the predilution step is carried out by diluting the serum sample about 50% by volume with a suitable isotonic diluent such as a saline solution. The resulting prediluted sample is combined (step (a)) with an isotonic solution containing a sphering agent (sometimes referred to herein as detergent). A typical first dilution results in a 50:1 dilution of sample. A further dilution (step (b)) is effected by treating an aliquot of the above sample with a protein-sphering agent solution preferably to provide a dilution of sample of about 1000:1. The resulting sample contains sphered and stabilized erythrocytes at a feasible concentration for light scattering measurement. When such light scattering measurement is conducted employing a flow cell cytometer, the individual cell volumes can be determined as well as the number of cells. The mean volume can therefore also be calculated.

A critical feature of the method of the invention is the weight ratio of protein/sphering agent and the concentration of sphering agent. By regulating these parameters within certain limits, the sphering process is effectively accomplished and the analytical results highly probative.

The weight ratio of protein/sphering agent is from about 20:1 to about 70:1, with a ratio of 50:1 being most preferred. For the final concentration of sphering agent, a concentration of from about 2 mg./100 ml. to about 10 mg./100 ml. is used, with a concentration of 3 mg./100 ml. most preferred.

The protein, externally supplied, is preferably a serum albumin. Other employable proteins include bovine, human and egg albumin.

In the modified method of this invention, the protein/sphering agent second dilution step (b) is replaced by treatment with an isotonic fixing agent solution (step (c)). In this system, the protein for the first dilution is the endogenous protein in the serum sample, i.e. the plasma protein. An isotonic solution of a sphering agent is added in a volume sufficient to bring the endogenous plasma protein/sphering agent ratio and also the concentration of sphering agent within the desired ranges. The preferred fixing agent is glutaraldehyde, used in an amount to provide a final glutaraldehyde concentration of from 0.1% to 0.4% by weight. The isotonic fixing agent solution is suitably formulated with saline or a saline-sphering agent mixture.

Because glutaraldehyde fixes red cells very rapidly, optimal buffering of the sphering agent concentration beyond the fixing agent addition step is considered less critical. As soon as the red cell content has been fixed, it becomes completely noncritical.

The sphering agent employed in either method is suitably an alkali metal (sodium, potassium, lithium, cesium or rubidium) salt of an alkyl sulfate wherein said alkyl contains from 10 to 16 carbons. Alkali metal lauryl sulfates are preferred, and sodium lauryl sulfate most preferred. Other suitable sphering agents which may be employed in these methods include fatty acids, phospholipids,

etc. It is to be noted that some nominal "sphering agents" such as crude egg lecithin (see Ponder above) actually contain the sphering agent only as a minor impurity. For example, pure lecithin is not a sphering agent. It is to be understood that the weight concentrations discussed are of the active principle in any impure "sphering agent" and not the crude weight concentration.

Both the methods of the invention can be effected either continuously as in an automated system, or in a discontinuous or discrete manner.

In order that the invention may be more fully understood, an embodiment thereof will now be described, by way of example only, with reference to the accompanying drawing, wherein the single Figure represents a schematic flow sheet of a continuous system for carrying out the method of the present invention for the treatment of a whole blood sample for eventual electrooptical measurement.

Referring to the drawing, a system is illustrated for measuring the volume of individual red cells in a discrete anticoagulated blood sample treated in accordance with the present invention. However, it is within the scope of the present invention that the measurement of the volume of red cells in successive anticoagulated blood samples may be effected on a continuous basis, for example, as described in our United States Patent No. 3,740,143.

The system comprises a peristaltic pump 1 including pump tubes 3,5,7,9 and 10. As will be understood, the relative internal diameters of the pump tubes determines the proportioning of the sample and reactants introduced into the system. An aspirating probe 13 is connected along conduit 14, to the inlet of pump tube 5, whose outlet is connected to a junction 15. Probe 13 is adapted to be immersed in an anticoagulated blood sample 17 contained in a sample receptacle 19. It will be appreciated that probe 13 may be adapted, as described in Untied States Patent

No. 3,740,143, to be immersed, in turn, into successive sample receptacles, so as to effect the measurement of the red cell volumes of successive samples on a continuous basis.

The inlet end of pump tube 3 is connected to a source 21 of appropriate diluent for effecting the first dilution of the sample 17. Upon operation of pump 1, diluent is passed along pump tube 3 to junction 23 in conduit 14, so as to be mixed with, and diluent, the sample being passed from the probe 13. Also, an air line 25 from an "air-bar" structure 26, as described in our United States Patent No. 3,306,229, whose operation is phased to that of the pump 1 as indicated by the dashed connector, operates periodically to introduce occluding air segments into conduit 14. The presence of such "intra-sample" air segments ensures proper proportioning of the sample and reactants into the system (and effective wash between successive samples) as described in the referenced patent. Concurrently, an isotonic solution containing the sphering agent is passed from source 27 along pump tube 7 to junction 15, whereat it is mixed with the diluted sample passed along pump tube 5, to effect the second dilution of sample 17. The sample is flowed from junction 15 and through mixing coil 29, to effect a thorough mixing thereof, and subsequently along conduit 31 to a resampling fitting 33. Fitting 33 includes a waste outlet 35 and a resampling outlet 37 connected to the inlet of pump tube 9. The sample passes from outlet 37 and to junction 39 along pump tube 9, excess sample and "intra-sample" air segments introduced into fitting 33 being passed to waste along waste outlet 35. A second "air-bar" structure 38 reintroduces "intra-sample" air segments along air line 36 into the diluted sample stream.

The inlet of pump tube 10 is connected to a source 41 of fixing agent. The outlet of pump tube 10 is connected

to junction 39, whereat the fixing agent and the twice-diluted sample are mixed and passed to mixing coil 43, to ensure mixing of the same. The outlet of mixed coil 43 is passed to a resampling fitting 45, which includes a waste outlet 47 and a resampling outlet 49, the latter being connected to the inlet of the single pump tube of a secondary peristaltic pump 51. The sample is passed from the outlet 49 and through pump 51 to a sheath-stream particle counter 53, of the type described in United States Patent No. 3,740,143. Again, excess sample and the "intra-sample" air segments are passed to waste along waste outlet 47. In counter 45, the red cells in the treated blood sample are confined to flow serially, so as to be individually counted and their volumes measured. The treated blood sample is thereafter passed to waste. The sphering of the red cells, according to the present invention, ensures that the measured volume is independent of the orientation of the red cells as they progress through counter 53. In prior art, where the red cells were not properly sphered, the random orientation of the red cells proceeding through the particle counter often resulted in inaccurate volume determinations.

The following Examples are given by way of illustration only.

EXAMPLE I

A sample (0.37 ml.) of anticoagulated whole blood is prediluted with isotonic saline (0.23 ml.). An aliquot (0.16 ml.) of the resulting sample is combined with 4.2 ml. of an isotonic saline solution containing sodium lauryl sulfate (3 mg./100 ml.). An aliquot (0.16 ml.) of the resulting diluted sample is then treated with 4.0 ml. of an isotonic saline solution containing bovine serum albumin (0.1%) and sodium lauryl sulfate (3 mg./100 ml.). The final sample is placed in a flow cell and electrooptically measured. The red blood cell count and red blood cell

volume were recorded.

EXAMPLE II

A sample (0.37 ml.) of anticoagulated whole blood is prediluted with isotonic saline (0.23 ml.). An aliquot (0.16 ml.) of the resulting sample is combined with 4.2 ml. of an isotonic saline solution containing sodium lauryl sulfate (3 mg./100 ml.). An aliquot (0.16 ml.) of the resulting diluted sample is then treated with 4.0 ml. of an isotonic saline solution containing glutaraldehyde (0.2%) and sodium lauryl sulfate (1 mg./100 ml.). The final sample is placed in a flow cell and electrooptically measured. The red blood cell count and red blood cell volume were recorded.

CLAIMS:

1. A method of treating mammalian whole blood to provide a sample suitable for electrooptical determination of red blood cell volume, which comprises the steps of (a) combining an anticoagulated whole blood sample with a first isotonic solution containing sphering agent, and (b) treating an aliquot of the resulting mixture with a second isotonic solution containing protein and sphering agent; wherein the weight ratio of protein to sphering agent in the aliquot and in the final sample is from about 20:1 to about 70:1, and the concentration of sphering agent in the final sample is from about 2 mg./100 ml. to about 10 mg./100 ml.

2. A modification of the method of claim 1, wherein instead of step (b), said aliquot from step (a) is treated in a step (c) with an isotonic solution containing fixing agent, and wherein the aliquot from step (a) has a weight ratio of endogenous protein to sphering agent of from about 20:1 to about 70:1, and a sphering agent concentration of from about 2 mg./100 ml. to about 10 mg./100 ml.

3. A method according to claim 2, wherein said fixing agent solution contains glutaraldehyde in an amount to provide a final glutaraldehyde concentration of from 0.1% to 0.4% by weight.

4. A method according to any preceding claim, wherein prior to step (a), said whole blood sample is prediluted with saline as diluent, resulting in about a 50% by volume dilution of sample.

5. A method according to claim 4, wherein step (b) or step (c) results in a further dilution of original

sample of about 1:1000 by volume.

6. A method according to any preceding claim, wherein said sphering agent is an alkali metal salt of an alkyl sulfate, said alkyl group containing from 10 to 16 carbon atoms.

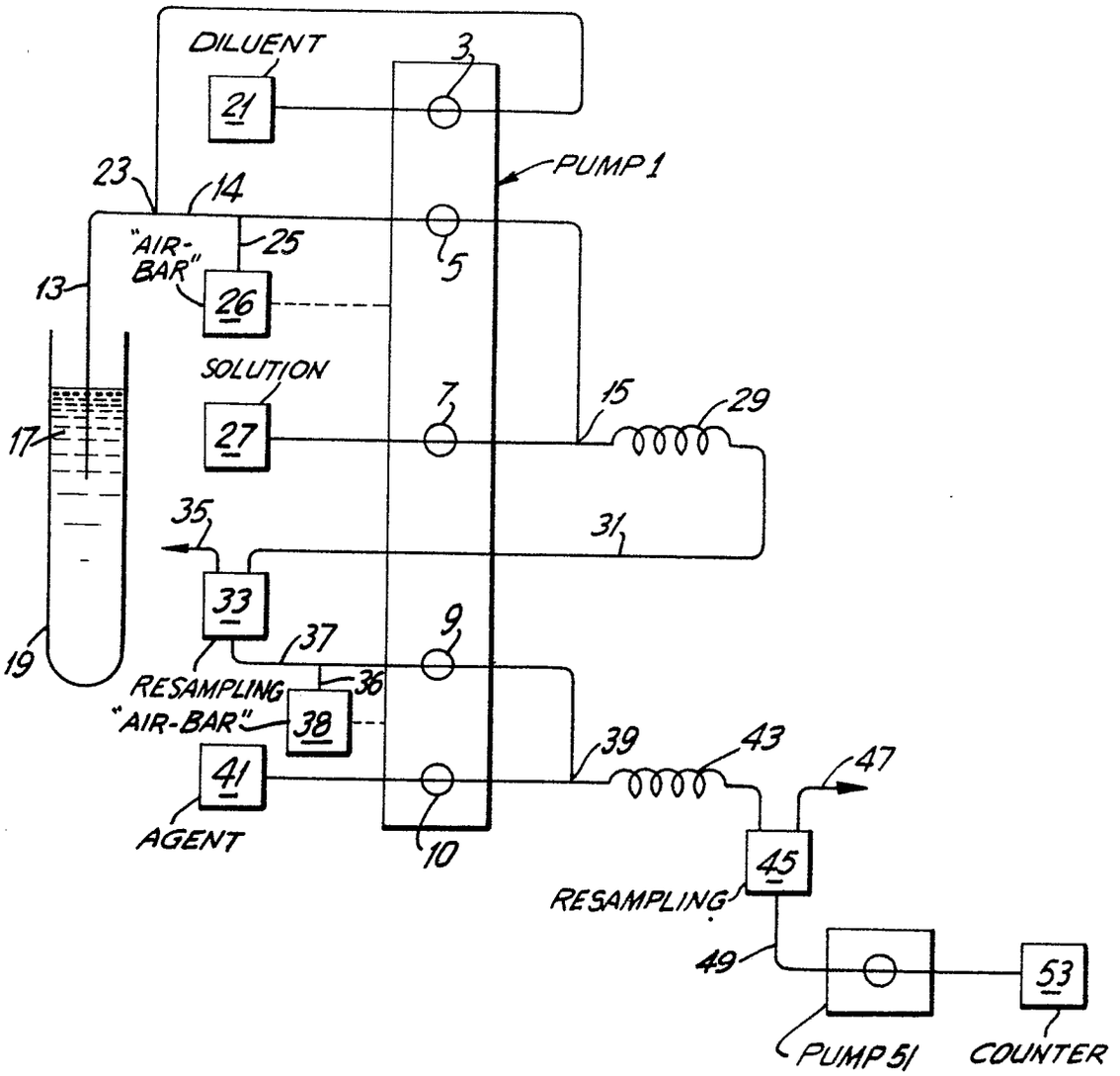
7. A reagent for sphering the red blood cells in an anticoagulated whole blood sample, which comprises a mixture of a protein and a sphering agent wherein the weight ratio of protein to sphering agent, when said reagent is mixed with a blood sample, is from about 20:1 to about 70:1, and wherein the total concentration of sphering agent is such as to provide, when said reagent is mixed with the blood sample, from about 2 mg./100 ml. to about 10 mg./100 ml. of sphering agent in the blood sample mixture.

8. A reagent according to claim 7, wherein said sphering agent is an alkali metal salt of an alkyl sulfate, said alkyl group containing from 10 to 16 carbon atoms.

9. A method of preparing stable, sphered and fixed mammalian red blood cells as calibration particles for flow cytometry which comprises the steps of (a) combining an anticoagulated whole blood sample with an isotonic solution containing sphering agent and (b) treating the resulting solution with a fixing agent solution, the product of step (a) having a weight ratio of endogenous protein to sphering agent of from about 20:1 to about 70:1, and a final concentration of sphering agent of from about 2 mg./100 ml. to about 10 mg./100 ml.

10. A reagent for stabilising, sphering and fixing red blood cells as calibration particles for flow cytometry

which comprises a sphering agent/fixing agent mixture wherein the protein/sphering agent weight ratio after mixing with whole blood is from about 20:1 to about 7:1 and a sphering agent concentration of from about 2 mg./100 ml. to about 10 mg./100 ml.


DILUENT
21
3
PUMP 1
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"AIR-BAR"
25
26
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SOLUTION
27
7
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17
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37
19
RESAMPLING "AIR-BAR"
38
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43
47
41
AGENT
10
45
RESAMPLING
49
53
PUMP 51
COUNTER

FIG. 1

European Patent Office

EUROPEAN SEARCH REPORT

Application number
EP 82302524.2

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim
A	DE - A - 2 340 252 (J.P. MERIC) * Page 7, 2nd paragraph; page 18, 2nd paragraph; claim 4 * --	1
A	DE - A - 2 250 363 (COULTER ELECTRONICS) * Claims 1-18, especially claims 8,9 * ----	1

CLASSIFICATION OF THE APPLICATION (Int. Cl. 3)

G 01 N 33/50
G 01 N 33/48//
G 01 N 15/02
G 01 N 15/06

TECHNICAL FIELDS SEARCHED (Int.Cl. 3)

G 01 N

CATEGORY OF CITED DOCUMENTS

X: particularly relevant if taken alone
Y: particularly relevant if combined with another document of the same category
A: technological background
O: non-written disclosure
P: intermediate document
T: theory or principle underlying the invention
E: earlier patent document, but published on, or after the filing date
D: document cited in the application
L: document cited for other reasons

&: member of the same patent family, corresponding document

X The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
VIENNA	13-09-1982	SCHNASS